

AD _____

Award Number: DAMD17-97-1-7036

TITLE: Restore Wild-Type Functions to P53 Mutants Using an
RNA-Based Combinatorial Approach

PRINCIPAL INVESTIGATOR: Christopher Green, Ph.D.

CONTRACTING ORGANIZATION: SRI International
Menlo Park, California 94025-3493

REPORT DATE: June 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010323 025

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2000	3. REPORT TYPE AND DATES COVERED Final (15 May 97 - 14 May 00)	
4. TITLE AND SUBTITLE Restore Wild-Type Functions to P53 Mutants Using an RNA-Based Combinatorial Approach			5. FUNDING NUMBERS DAMD17-97-1-7036	
6. AUTHOR(S) Christopher Green, Ph.D.			8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) SRI International Menlo Park, California 94025-3493 E-MAIL: christopher.green@sri.com				
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The p53 protein is a transcription factor and a suppressor regulating the expression of a wide range of genes involved in apoptosis, growth control, and inhibiting the proliferation of tumor cells in animal models. Mutations that inactivate the p53 gene are important steps in tumor progression and often affect the protein's DNA binding ability. Attempts were made to select RNA molecules that bind to human p53 protein or a small peptide from its carboxyl terminus to determine whether these could be used to alter the protein's ability to bind the p53 consensus DNA-binding sequence. These attempts were unsuccessful, however the experience gained will be applied to another somewhat easier targets involved in tumor growth.				
14. SUBJECT TERMS Breast Cancer, CDA Award, p53, SELEX			15. NUMBER OF PAGES: 31	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

CR Where copyrighted material is quoted, permission has been obtained to use such material.

CR Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

CR Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Christopher J. Green June 12, 2000
PI - Signature Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	21
Reportable Outcomes.....	22
Conclusions.....	23
References.....	25
Appendices.....	27

INTRODUCTION

The objective of this research was to produce a nucleic acid sequence that will bind to mutant human p53 protein to enhance its tumor suppressor ability. Normal p53 protein is both a transcription factor and a suppressor regulating the expression of a wide range of genes involved in apoptosis, growth control, and inhibition of tumor cells proliferation in animal models. Mutations that inactivate the p53 gene are important steps in tumor progression and often affect the protein's DNA binding ability. These mutant p53 proteins have significant changes in their structure (for a review, see Erlanson and Verdine, 1994). Inactivation of the p53 protein occurs often in advanced breast cancer, and since loss of p53 functions results in increased cell proliferation and decreased cell death, tumors without a functioning p53 are more likely to have a very aggressive clinical course (Elledge et al., 1993). If a method can be found to return the structure of a cancer cell's mutant p53 to more more nearly resemble the wild-type form, the cell's normal apoptotic mechanisms may be activated, resulting in the cell's self-destruction. We attempted to use SELEX (Systemic Evolution of Ligands by EXponential enrichment) (Fitzwater and Polisky, 1996; Conrad and Ellington, 1996; Tuerk and Gold, 1990) to find short RNA molecules that can bind to recombinantly produced p53. The use of specific nucleic acid sequences to bind to p53 protein to accomplish this task has some significant advantages. Combinatorial methods can be used to screen a large number of possible RNA sequences and once effective RNA oligomers are found, it is conceivable that viral vectors can be constructed that can infect cells and express the desired RNA to restore normal function to mutant p53 protein.

BODY

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF HUMAN WILD TYPE P53 PROTEIN

The human p53 expression system used was provided by Dr. Peter Tegtmeier of the State University of New York at Stony Brook. The vector contained a human wild type p53 gene 1.3 kb (hWtp53) that had been inserted into a plasmid (pCMH6K) downstream of a sequence for a typical mammalian translation initiation sequence and a sequence coding for the following amino acids: MAYPYDVPDYAARHHHHHH. This peptide leader codes for a hemagglutinin epitope followed by six histidine residues that allow for rapid purification by Ni-affinity chromatography. This expression cassette was then excised using unique *Nhe* I and *Bam*H I sites and subcloned into a pIT baculovirus expression vector (Wang et al., 1993).

Large scale expression was carried out in three T150 tissue culture flasks. The cells were harvested and lysed in buffer containing 150 mM Tris-HCl at pH 9.0, 150 mM NaCl, 0.5% NP-40 detergent, 10% glycerol, 2 μ M phenylmethylsulfonyl fluoride, 50 μ g/ml aprotinin, 50 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 2 mM benzamidine, and 1 mM β -mercaptoethanol at 4 °C. Cell lysates were passed through a Ni-NTA agarose column (Qiagen, Inc.) also at 4 °C and washed with 50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol, pH 6.0, until the optical density at 280 nm of the flow-through was less than 0.01. The p53 protein was eluted with 0.5 M imidazole in 50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol. The eluted p53 protein was dialyzed against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 50% glycerol at 4 °C overnight. The purity of the His-tagged recombinant p53 peptide was determined by polyacrylamide-sodium dodecyl sulfate gel electrophoresis and staining with Coomassie blue.

An electrophoretic mobility shift assay (EMSA) (Wang et al., 1993) was used to determine that the purified recombinant p53 peptide retained its specific DNA binding activity. Two double-stranded DNAs were synthesized for this work:

Consensus p53 binding DNA	5' -GGACATGCCCCGGGCATGTCC-3'
	3' -CCTGTACGGGCCCCGTACAGG-5'
Competitor DNA:	5' -TTGTTT'TTGGCAGTGTGCCAACAGTCG-3'
	3' -AACAAAAACCGTCACACGGTTGTCAGC-5'

The first double-stranded DNA is the consensus binding sequence for p53 (Funk et al., 1992). The second was used as a competitor for the binding to demonstrate specificity. In the EMSA shown in Figure 2, both of the double stranded DNAs were labeled at their 5'-ends with ^{32}P by using polynucleotide kinase. Each binding reaction used two ng (2×10^4 cpm) of the probes. The binding was performed in 20 μl of 20% glycerol, 25 mM HEPES, pH 7.4, 50 mM KCl, 1 mM dithiothreitol (DTT), 0.1% NP-40, 1 mg/ml bovine serum albumin at room temperature for 30 min with various amounts of the recombinant p53 and antibodies (Figure 2). The reactions were then run on a 4% native polyacrylamide gel electrophoresis. The anti-p53 antibodies PAb 421 and PAb 1801 (Hupp and Lane, 1992) were used to show that the recombinant peptide could be recognized by antibodies and a non-p53-specific antibody (anti-cdc2) was used to demonstrate that the antibody reaction was specific. The results indicate that the recombinant His-tagged p53 peptide retains its ability to specifically recognize its DNA binding site.

CONSTRUCTION OF THE RNA LIBRARY

A large pool of DNA templates were synthesized that contained a randomized 40-nucleotide-long sequence flanked by defined sequences. These defined sequences consisted of a 5'-polymerase chain reaction primer hybridization site that contained a T7 RNA polymerase promoter and a 3'-PCR hybridization site (Figure 3). Although the use of a 40-base-long random sequence would, in theory, produce 1.2×10^{24} different molecules (4^{40}), the actual mass of a complete collection of 40-residue-long DNA molecules would amount to 2 mol, or approximately 27 kg of DNA. Since only 1 μg of DNA template was used in PCR reactions to generate the double-stranded DNA library that was then used to make the RNA library, the actual number of different RNA molecules was approximately 10^{14} . The sequences of the DNAs used to make the

library are shown in Figure 4. Double stranded DNA (dsDNA) molecules were amplified by standard PCR reactions and purified on an 8% polyacrylamide gel. The purified dsDNA templates were then transcribed by the T7 RNA polymerase kit from Epicentre Technologies in 40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, and 1 mM of each of the four ribonucleotide triphosphates (dNTPs) to yield the initial pool of 92 nucleotide-long RNAs.

***IN VITRO* SELECTION OF RNA APTAMERS THAT BIND TO THE HIS-TAGGED P53 PROTEIN**

A nitrocellulose filter binding assay (Gold et al., 1995; Conrad and Ellington, 1996; Keene, 1996) was used to select RNA aptamers that bound to the p53 protein. The initial pool of 20 g of RNA was dissolved in 200 µl of binding buffer (Tian et al., 1995) consisting of 25 mM HEPES, pH 7.4, 50 mM KCl, 1 mM DTT, 1 mM Mg₂Cl, 0.2 mM EDTA, 10% glycerol, and 0.5 mM PMSF. The RNA solution was then heated to 94° C for 3 min and cooled to 4° C. To remove possible filter-binding RNA molecules, before each round of selection the RNA solution was passed through a 13-mm, 0.45-µm pore size Millipore HAWP cellulose acetate-cellulose nitrate membrane on a filter holder on a vacuum manifold (J. T. Baker) under a pressure of 5 inches of Hg (Conrad and Ellington, 1996). One microgram of the pure recombinant p53 protein was added to the RNA in the binding buffer and the mixture incubated at room temperature for 1 h. This p53-RNA solution was then passed through the same type of filter and washed twice with 200 µl of the same binding buffer. The RNA bound to p53 on the filter was eluted by suspending the filter in 200 µl of 7 M urea and extracting with 600 µl of phenol-chloroform at pH 8.0. The eluted RNA was then ethanol precipitated in the presence of 5 µg of glycogen and redissolved in 20 µl of RNase-free water.

CONVERSION OF THE SELECTED RNAS INTO NEW DNA TEMPLATES

The selected RNAs were transcribed into cDNA by using the 3'-primer shown in Figure 3 and avian myeloblastosis virus reverse transcriptase (AMV-RT) in a total volume of 50 µl of 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM MgCl₂, 4 mM DTT, 200 µM each of the four dNTPs,

and 1 μ M of the 3'-primer on a thermocycler. Before the AMV-RT enzyme was added, the solution was denatured at 75° C for 3 min and renatured at 25° C for 5 min. Then 20 units of AMV-RT was added and incubated first for 30 min at 37° C and then for a further 15 min at 48° C (Fitzwater and Polisky, 1996). The cDNA was ethanol precipitated in the presence of 5 μ g of glycogen. This material was then amplified by a standard PCR reaction using two reaction tubes, each containing 100 μ l; this volume comprised one half of the above cDNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, and 1 μ M of each of the two primers shown in Figure 3. The thermocycler conditions were a preliminary denaturation for 3 min at 94° C and then 25 cycles of 94° C for 45 s, 55° C for 45 s, and 72° C for 1 min. The reaction was then incubated for a further 7 min at 72° C. A new pool of RNAs for the next round of selection was then generated using the T7 RNA polymerase kit described above.

ANALYSIS OF RNAS SELECTED FROM THE SELEX PROCEDURE

The selection procedure was repeated for 10 cycles. After the tenth round, the RNA was converted one last time into dsDNA, which was then cloned into the pCRVector plasmid by using the Invitrogen TA Cloning Kit. Eighteen plasmid clones were purified and sequenced using an automated sequencer. Six different sequences were found and are shown in Figure 4. The secondary structure and thermodynamic stability predictions made for these sequences by the "MulFold" program (Jaeger et al., 1989) are shown in Figure 5.

The plasmid clones described above were used as PCR templates with the primers shown in Figure 3 to generate the dsDNA that were used for T7 RNA RNA polymerase transcription of the pure RNAs. The resulting RNAs were quantitated by spectrophotometry. These RNAs were dissolved to a concentration of 50 ng/ μ l in 25 mM HEPES, pH 7.4, and 50 mM KCl and heated at 94° C for 3 min and then cooled to 4° C. The effect of the selected p53-binding RNAs was studied by electrophoretic mobility shifts as described above. The results (Figure 6 and 7) indicate that all of the selected RNAs interfere with the sequence specific DNA binding activity of p53, but with a wide difference in the extent of inhibition by the various clones. RNA#12 demonstrated the least interference with sequence specific DNA binding activity of p53. There was no correlation between activity and thermodynamic stability.

ATTEMPT TO SELECT RNAS THAT BIND TO THE CARBOXYL TERMINUS OF P53

The carboxyl terminus of the p53 protein has been demonstrated to negatively regulate the protein's specific DNA binding ability. A synthetic 22-mer peptide from the carboxyl terminus (residues 361-382) of p53 protein can restore the DNA binding and transcriptional activity of some mutant p53 proteins (Selinova et al., 1997). Antibodies directed at the carboxyl terminus of p53 protein have been shown to enhance the specific DNA binding ability of wild type p53 protein and to restore the ability of mutant p53 protein to bind DNA (Hupp et al., 1992; Halazonetis and Kandil, 1993; Niewolik et al., 1995; Abarzua et al., 1996). When single-chain fragments derived from monoclonal antibodies directed against p53 were expressed in human tumor cells, the cell's mutant p53 protein transcriptional activity was restored *in vivo* (Fromentel, et al., 1999).

Rather than using the entire p53 protein molecule as a target for the selection of RNA oligomers, a synthetic 22-mer peptide was purchased from Bachem Bioscience, Inc. This peptide corresponds to residues 361-382 of the carboxyl terminus of human p53 protein (see Figure 8). Since this peptide is positively charged, it makes a good candidate for the use of combinatorial methods to select a RNA oligomer. The template used to produce the random RNA oligomer was the same as used in the earlier attempt against whole p53 (Figure 3). The T7 RNA polymerase template containing a 40-base-long random sequence was used to generate a ³²P-labeled set of RNA oligomers. This RNA was then incubated with the 22-mer p53 peptide in 20 µl of 25 mM HEPES at pH 7.4, 50 mM KCl, 1 mM DTT, 1 mM Mg₂Cl, and 0.2 mM EDTA for 1 h at room temperature and electrophoresed through a 5% nondenaturing acrylamide gel. Any RNA that binds to the peptide is retarded by the net reduction in charge and the increase in the size of the complex. The positions of the radiolabeled RNA were detected by phosphor imaging on a Molecular Dynamics Storm 840 (Figure 9 for an example). The RNA that was retarded by binding to the peptide is excised from the gel and eluted. This RNA that had been enriched for species that bind to the p53 peptide was then converted back into a dsDNA T7 RNA polymerase template by reverse transcriptase-PCR (RT-PCR) using the primers shown in Figure 3. This new template was then used again to generate more RNA for another round of selection. This procedure can be repeated any number of cycles.

After at least five cycles had been completed, the dsDNA product from the RT-PCR reaction was then ligated into a pBluescript plasmid vector (Life Technologies) and the resulting clones sequenced using an Applied Biosystems 310 automated capillary electrophoresis sequencer. The sequences were examined for common features, and individual clones were selected for RNA transcription and determination of their relative binding abilities to the p53 peptide.

Unfortunately, this procedure, even when put through 10 cycles, did not result in RNA species that showed any better binding than the original unselected RNA library. The library that had been put through 10 selection cycles was converted back into dsDNA and cloned into pCR-Script plasmids. Twenty clones were sequenced on an Applied Biosystems model 310 Genetic analyzer to see if there were any common features, but none could be found. Individual clones that were converted back into RNA did not show any better binding to the p53 peptide than the original unselected mixture.

10 20 30 40 50 60 70 80 90 100
 GCTAGCCATGGCTTACCCATACGACGTACCAGACTACGCAGCGCGACATCATCATCATCATGCGCGCCGAGCATCAGTAGGGGTTATGGAGGAGCCG
M A Y P Y D V P D Y A A R H H H H H A R R A S V G V M E E P>
 110 120 130 140 150 160 170 180 190 200
 CAGTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTTCAGACCTATGGAACTACTTCCTGAAAAACAACGTTCTGTCCCCCTTGCCGTCCC
 Q S D P S V E P P L S Q E T F S D L W K L L P E N N V L S P L P S>
 210 220 230 240 250 260 270 280 290 300
 AAGCAATGGATGATTTTGATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGATGAAGCTCCAGAAATGCCAGAGGCTGCTCC
 Q A M D D L M L S P D D I E Q W F T E D P G P D E A P R M P E A A P>
 310 320 330 340 350 360 370 380 390 400
 CCCCCTGGCCCCCTGCACACGAGCTCCTACACCGCGCCCTGACACAGCCCCCTCTGGCCCCCTGTATCTTCTGTCCCTTCCCAGAAAACCTACCAG
 P V A P A P A P T P A A P A P A P S W P L S S S V P S Q K T Y Q>
 410 420 430 440 450 460 470 480 490 500
 GGCAGCTACGGTTTCCGTCTGGGCTTCTTGCACTTCTGGGACAGCCAAGTCTGTGACTTGCACTGCTCCCTGCCCTCAACAAGATGTTTGGCAACTGG
 G S Y G F R L G F L H S G T A K S V T C T Y S P A L N K M F C Q L>
 510 520 530 540 550 560 570 580 590 600
 CCAAGACCTGCCCTGTGACGTGTGGGTTGATTCACACCCCCCGCCCGCACCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCAGATGACCGA
 A K T C P V Q L W V D S T P P P G T R V R A M A I Y K Q S Q H M T E>
 610 620 630 640 650 660 670 680 690 700
 GGTGTGAGGCGCTGCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGGCCCCCTCTCAGCATCTTATCCGAGTGAAGGAAATTTGCGTGTGGAG
 V V R R C P H H E R C S D S D G L A P P Q H L I R V E G N L R V E>
 710 720 730 740 750 760 770 780 790 800
 TATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTCCCTATGAGCCGCTGAGGTTGGCTCTGACTGTACCACCATCCACTACAACATCATGT
 Y L D D R N T F R H S V V V P Y E P P E V G S D C T T I H Y N Y M>
 810 820 830 840 850 860 870 880 890 900
 GTAACAGTTCTGTCATGGGCGCATGAACCGGAGGCCCATCTCACCATCATCACTGGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGA
 C N S S C M G G M N R R P I L T I I T L E D S S G N L L G R N S F E>
 910 920 930 940 950 960 970 980 990 1000
 GGTGCATGTTTGTGCTGTCTGGGAGAGACCGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGAGCCTCACCACGAGCTGCCCCAGGGAGCACT
 V H V C A C P G R D R R T E E E N L R K K G E P H H E L P P G S T>
 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
 AAGCGAGCACTGCCCAACAACACGAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTACCCCTTCAGATCCGTGGGCGTGAGCGCT
 K R A L P N N T S S S P Q P K K K P L D G E Y F T L Q I R G R E R>
 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 TCGAGATGTTCCGAGAGCTGAATGAGGCCTTGAACCTCAAGGATGCCCAGGCTGGGAAGGAGCCAGGGGGAGCAGGGCTCACTCCAGCCACCTGAAGTC
 F E M F R E L N E A L E L K D A Q A G K E P G G S R A H S S H L K S>
 1210 1220 1230 1240 1250 1260 1270
 CAAAAAGGGTCAGTCTACCTCCCGCCATAAAAACTCATGTTCAAGACAGAAGGGCCTGACTCAGACTGAGGGATCC
 K K G Q S T S R H K K L M F K T E G P D S D *>

Figure 1. The sequence of the expression cassette for human p53 with a hemagglutinin-His-tagged leader
 (underlined).

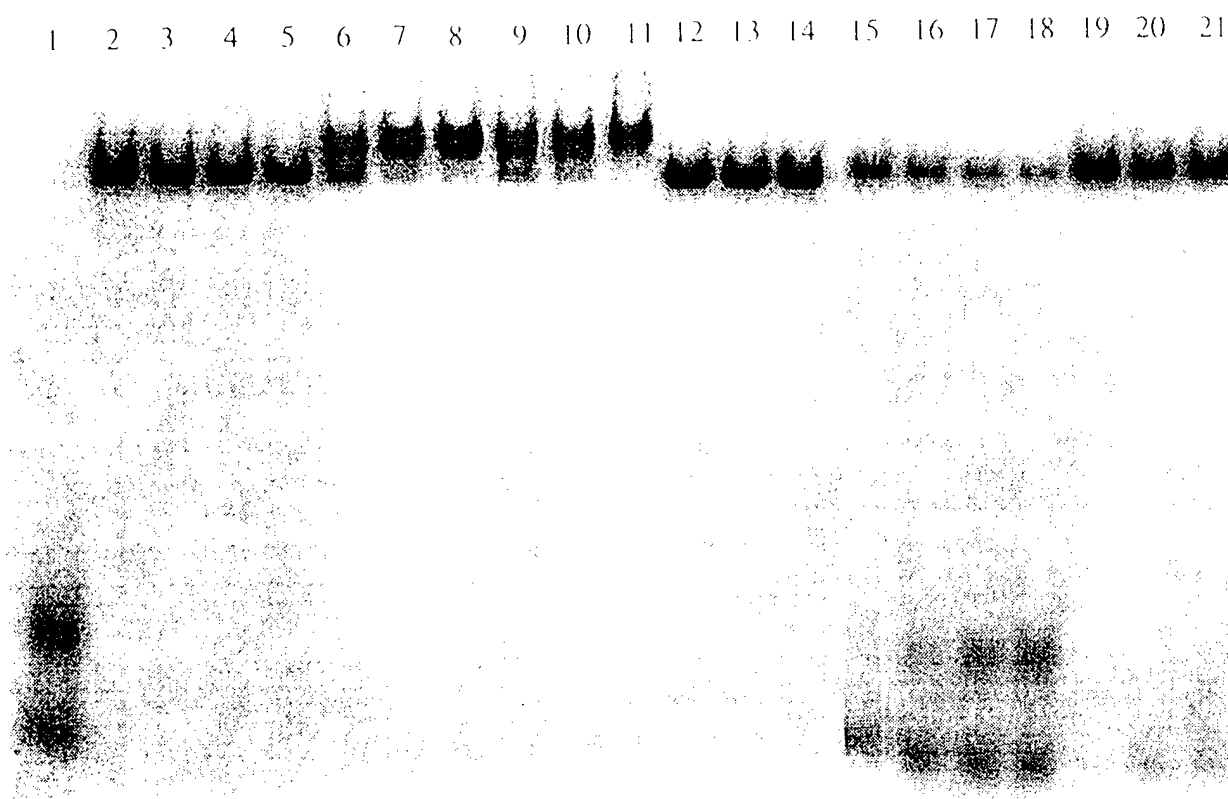


Figure 2. Electromobility shift assay of the purified human His-tagged p53 protein, using the ^{32}P -labeled consensus p53-binding DNA probe and a 4% polyacrylamide native gel.

Lane 1, ^{32}P -labeled consensus p53-binding DNA probe alone.

Lanes 2-5, 1, 0.5, 0.25, 0.1 μg p53, respectively.

Lanes 6-8, 0.5 μg p53 plus 0.1, 0.2, and 0.3 μg of p53-specific antibody PAb421, respectively.

Lanes 9-11, 0.5 μg p53 plus 0.1, 0.2, and 0.3 μg of p53-specific antibody PAb1801, respectively.

Lanes 12-14, 0.5 μg p53 plus 0.1, 0.2, and 0.3 μg of a anti-cdc2 antibody, respectively.

Lanes 15-18, 0.5 μg p53 plus 0.02, 0.05, 0.10, and 0.20 μg of unlabeled consensus p53-binding DNA probe, respectively.

Lanes 19-21, 0.5 μg p53 plus 0.02, 0.05, 0.10, and 0.20 μg of unlabeled nonspecific DNA probe, respectively.

5' TAATACGACTCACTATAGGGCGAATTCGGGTT (N)₄₀CCCTTTAGTGAGGGTTAATT
3' ATTATGCTGAGTGATATCCCGCTTAAGCCCAA (N)₄₀GGGAAATCACTCCCAATTAA

5'-primer 5' TAATACGACTCACTATAGGGCGAATTCGGGTT

3'-primer 5' AATTAACCCTCACTAAAGGG

Figure 3. T7 transcription template for RNA oligomer and PCR primers. The T7 promoter is underlined.

RNA #3

5' GGGCGAAUUCGGGUUUGGUAUGCGGGGUACUAAUAGUCGAGUGUGUUGUUCUCCCUUUAGUGAGGGUAAUU-3'

RNA #6

5' GGGCGAAUUCGGGUUAUGGUGGUACUGAUGGUGGUUCUCGUUGCAGUCCACGUGUCCCUUUAGUGAGGGUAAUU-3'

RNA #9

5' GGGCGAAUUCGGGUUUUUGGUAGUGGAGGUAGGUACUGUGGUUUUGUCCGUCGGUCCCUUUAGUGAGGGUAAUU-3'

RNA #12

5' GGGCGAAUUCGGGUUGGAUAGUAGGCGCAUAUGGCAUCUUCGUGGUUGUGUAUUGCCCUUUAGUGAGGGUAAUU-3'

RNA #15

5' GGGCGAAUUCGGGUUUUUAGGGAGGGACUAAUUUAAGAGAGUCGCCGUCGGUGUCCCUUUAGUGAGGGUAAUU-3'

RNA #17

5' GGGCGAAUUCGGGUUUUGUAGUGGUGGAGGAGGUUCUCGGUUCGUGCGUGGCUAGCCCUUUAGUGAGGGUAAUU-3'

Figure 4. p53-binding RNAs

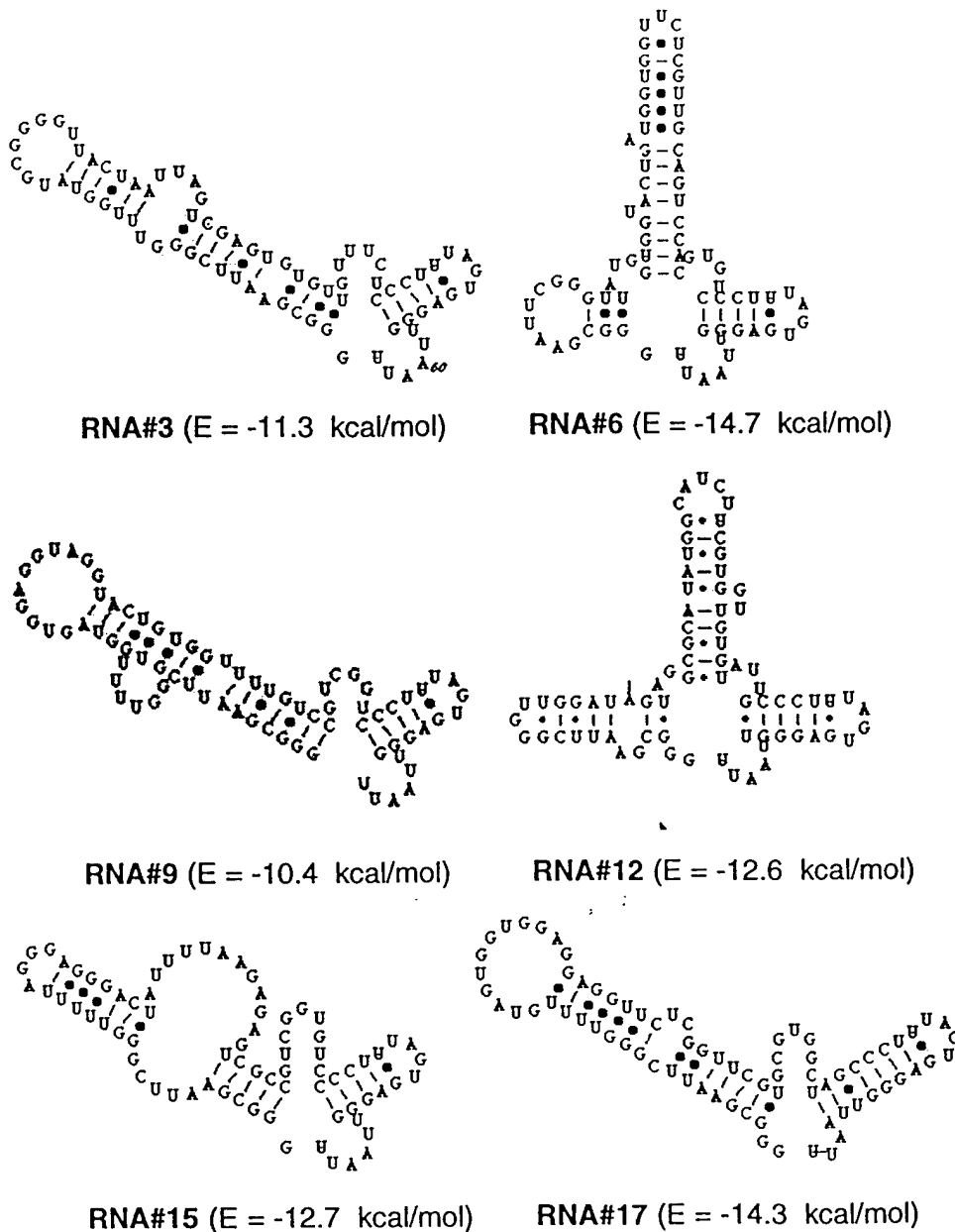


Figure 5. The secondary structures and thermodynamic stabilities (E) of the p53-binding RNAs predicted by the MulFold program.

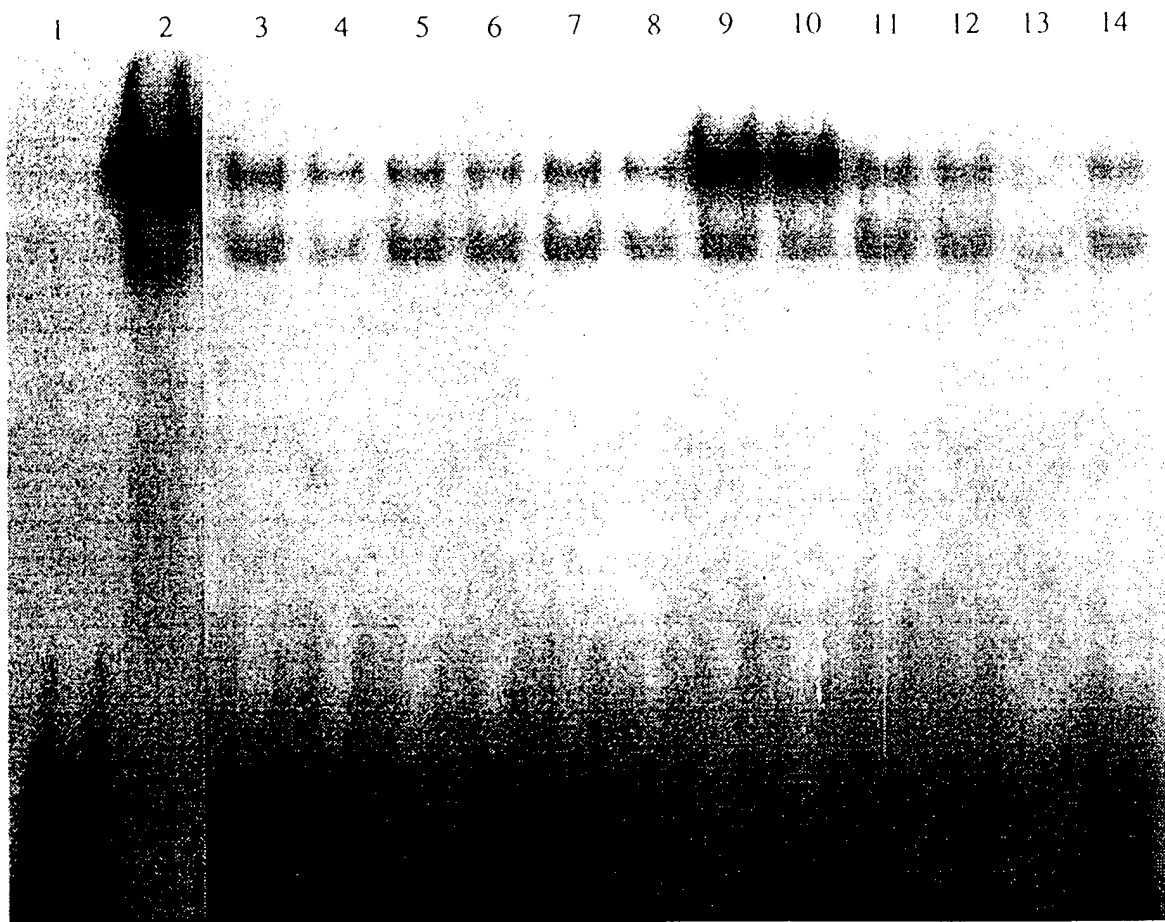


Figure 6. The effect of selected RNAs on the sequence-specific binding activity of human wild-type p53 protein as measured by electromobility shifts on a 4% native polyacrylamide gel electrophoresis. The procedure and labeled probe were the same as in Figure 2.

Lane 1, ^{32}P -labeled consensus p53-binding DNA probe alone.

Lane 2, 0.3 μg p53, no RNA

Lanes 3-4, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#3, respectively

Lanes 5-6, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#6, respectively

Lanes 7-8, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#9, respectively

Lanes 9-10, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#12, respectively

Lanes 11-12, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#15, respectively

Lanes 13-14, 0.3 μg p53 plus 0.05 and 0.1 μg RNA#17, respectively

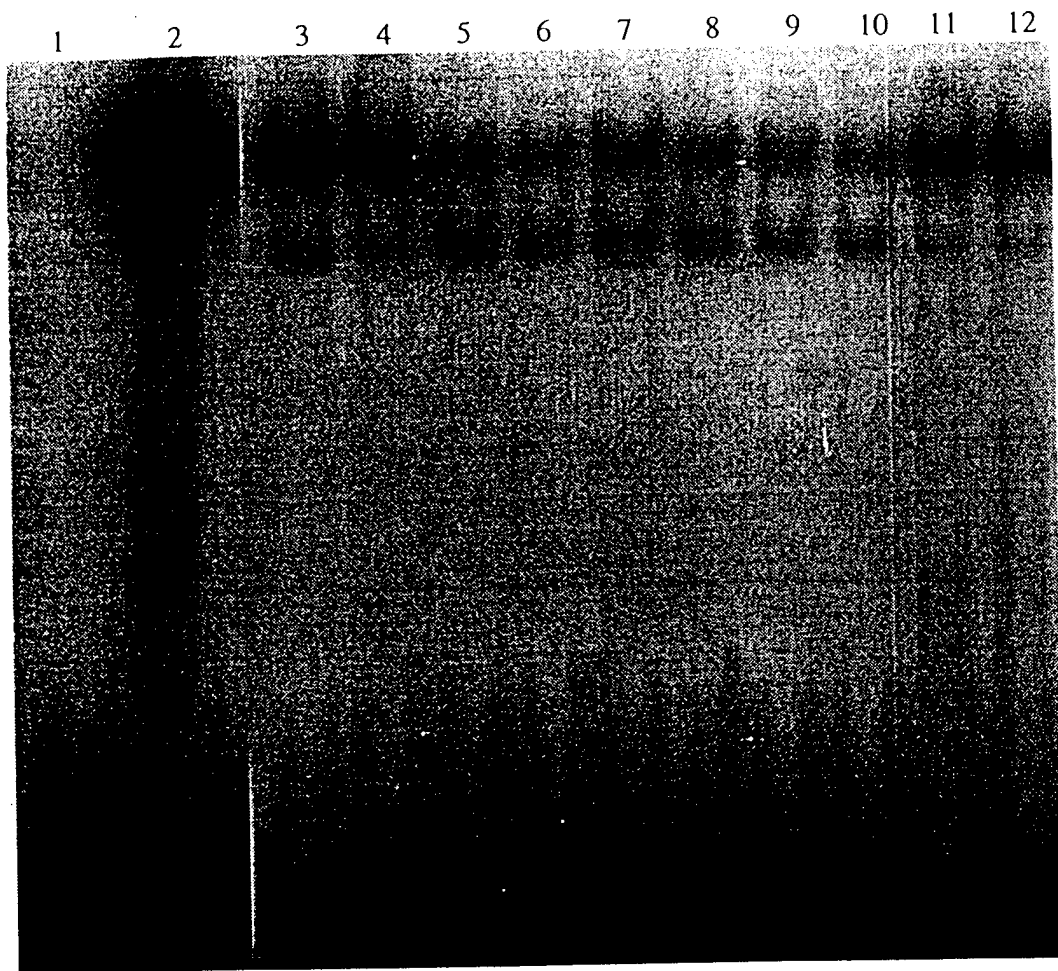


Figure 7. The effect of selected RNAs and tRNA^{Phe} on the sequence-specific binding activity of human wild-type p53 protein as measured by electromobility shifts on a 4% native polyacrylamide gel electrophoresis. The procedure and labeled probe was the same as for Figure 2.

Lane 1, ³²P-labeled consensus p53-binding DNA probe alone.

Lane 2, 0.2 µg p53, no RNA.

Lanes 3-4, 0.2 µg p53 plus 0.1 and 0.2 µg RNA#12, respectively.

Lanes 5-6, 0.2 µg p53 plus 0.1 and 0.2 µg RNA#17, respectively.

Lanes 7-8, 0.2 µg p53 plus 0.1 and 0.2 µg RNA#15, respectively.

Lanes 9-10, 0.2 µg p53 plus 0.1 and 0.2 µg RNA#3, respectively.

Lanes 11-12, 0.2 µg p53 plus 0.1 and 0.2 µg tRNA^{Phe}, respectively.

**H-Gly-Ser-Arg-Ala-His-Ser-Ser-His-Leu-Lys-Ser-
Lys-Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-Lys-Lys-OH**

Figure 8. Target peptide corresponds to residues 361-382 of the human p53 protein.

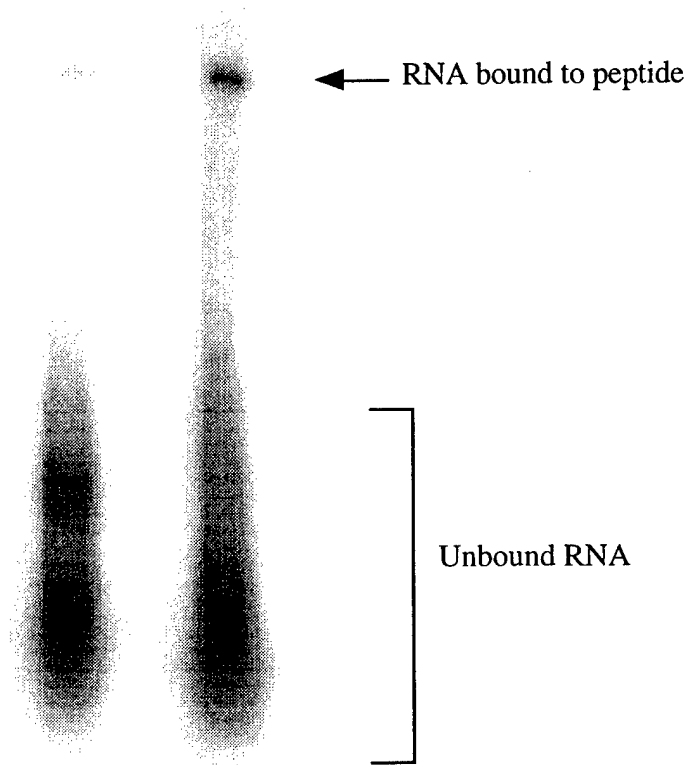


Figure 9. Nondenaturing gel electrophoresis of the fifth round of selection of RNA sequences that bind to the p53 22-mer peptide. The left lane contained the RNA without any peptide, and the right lane contained 100 ng of the peptide

KEY RESEARCH ACCOMPLISHMENTS

- A large amount of recombinant human p53 protein was produced that had a six-histidine-residue-long peptide leader.
- The recombinant p53 was purified and shown to be capable of specifically binding to its known double stranded consensus DNA sequence.
- A library of DNA containing a random stretch of 40 residues was synthesized and used to generate the random RNA library.
- The RNA library was put through ten rounds of selection for binding to the recombinant p53 protein.
- The DNA templates for the selected RNAs were cloned and sequenced.
- p53 binding of the selected RNAs was measured by electromobility shift gel assay.
- The RNA library was used to attempt the selection of species that could bind to a peptide from the carboxyl terminus of p53.

REPORTABLE OUTCOMES

The project has not resulted in a publication. The techniques that we developed will be used to support a NIH grant application.

CONCLUSIONS

The recombinant His-tagged p53 protein was expressed and purified in large amounts by use of the baculovirus system in insect cell culture. This peptide was shown to be capable of specific binding to a p53 consensus DNA binding sequence. This material was successfully used to select RNA species that could bind to the p53. The attempt to select a RNA to bind to the recombinant His-tagged p53 succeeded. However, the more difficult challenge of finding a particular RNA that could effect a significant change on p53 was not successful. Attempts to select a RNA to bind to the peptide from the critical region of the carboxyl terminus of p53 also did not succeed. The overall goal of the project was probably too ambitious since the selected p53-binding RNA molecules each would have little chance of having the desired effect of changing the structure of p53 significantly. It should be far easier to find RNAs that interfere with an important interaction rather than promote one.

The use of SELEX methods to find a nucleic acid to bind to an important component of the cells machinery may more profitably be employed against a relatively newly discovered protein, hypoxia-inducible factor-1 (HIF-1). The techniques that we have acquired for this p53 project will be used against this new target. HIF-1 is a transcription factor whose expression is tightly controlled by oxygen levels. The oxygen levels of relatively poorly vascularized tumor tissue usually leads to hypoxia (Semenza, 1998, Tacchini et al., 1999, Guillemin and Krasnow, 1997). This condition causes the induction of the expression of a number of genes, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) that promote angiogenesis to increase the flow on oxygenated blood to the growing tumor (Mustonen and Alitalo, 1995, Risau, 1997, Hanahan and Folkman, 1996, Ferrara, 1999). The induction of these genes is caused by the HIF-1 transcription factor. HIF-1 is a heterodimer made up by the HIF-1 alpha and HIF-1 beta (Wang and Semenza, 1995). The HIF-1 beta was recently identified as the previously known aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al., 1995).

Interfering with the interaction between the two components of HIF-1 should prevent the active hypoxia transcription factor complex from forming. This in turn should prevent hypoxic cells from inducing their normal response to low oxygen conditions. If the same techniques described above can be used to select RNA molecules that can interfere with the HIF-1 subunit

interaction, it could lead to new approaches to anticancer treatments by reducing the supply of nutrients to a tumor. An RNA that can interfere with the binding of two peptides should be inherently easier than trying to find an RNA that restores normal functioning to p53.

Our laboratories have expression vectors for both HIF-1 alpha and beta. We have also cloned the HIF-1 alpha cDNA into a histidine-tagged fusion protein expression vectors that can be used in mammalian and bacterial hosts. The histidine-tagged version of HIF-1 alpha has the same six histidine residues near the amino terminus as the p53 used for the work described above and allows for a similar purification procedure. We have used the bacterial expression vector to make large amounts of purified HIF-1 alpha in *E. coli*. We have also shown that the mammalian expression vector produces histidine-tagged HIF-1 alpha capable of forming an active HIF-1 complex with ARNT *in vivo* despite the presence of the amino terminal fusion peptide. We are now applying for grants to support further work in this area based on our experience gained on the p53 project.

REFERENCES

- Arbuzua, P. et al. (1995) *Cancer Research*, **55**, 3490-3494.
- Conrad, R., and Ellington, A. D. (1996) *Anal. Biochem.* **242**, 261-265.
- Elledge, R. M., et al., *Breast Cancer Res. Treat.* **26**, 225-235.
- Erlanson, D. A., and Verdine, G. L. (1994) *Chemistry & Biology*, **1**, 79-84.
- Ferrara, N. (1999) *J. Mol. Med.* **77**, 527-543.
- Fitzwater, T. and Polisky, B. (1996) *Methods Enzymol.* **267**, 275-301.
- Fromentel, C. C. et al. (1999) *Oncogene* **18**, 551-557.
- Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992) *Mol. Cell. Biol.* **12**, 2866-2871.
- Gold, L. (1995) *J. Biol. Chem.* **270**, 13581-13584.
- Guillemin, K., and Krasnow, M. A. (1997) *Cell* **89**, 9-12.
- Halazonetis, T. D., and Kandil, A. N. (1993) *EMBO J.*, **12**, 5057-5064.
- Hanahan, D., and Folkman, J. (1996) *Cell* **86**, 353-364.
- Hupp, T. R., and Lane, D. P. (1992) *Cell* **71**, 875-886.
- Jaeger, J. A., Turner, D. H. and Zucker, M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7706-7710.
- Keene, J. D. (1996) *Methods Enzymol.* **267**, 367-383.
- Niewolik, D. et al. (1995) *Oncogene* **10**, 881-890.
- Mustonen, T., and Alitalo, K. (1995) *J. Cell. Biol.* **129**, 895-898.
- Risau, W. (1997) *Nature* **386**, 671-674.
- Selinova, G. et al. (1997) *Nature Med.*, **3**, 632-638.
- Semenza, G. L. (1998) *Curr. Opin. Genet. Dev.*, **8**, 588-594.
- Tacchini, L. et al., *J. Biol. Chem.* **274**, 24142-24146.
- Tian, Y., et al., (1995) *RNA* **1**, 317-326.
- Tuerk, C., and Gold, L. (1990) *Science* **249**, 505-510.
- Wang, G. L. et al., (1995) *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 5510-5514.

Wang, Y., Reed, M., Wang, P., Stenger, J. E., Mayr, S., Anderson, M. E., Schwedes, J. F.,
and Tegtmeyer, P. (1993). *Genes Dev.* **7**, 2575-2586.

Wang, G. L., and Semenza, G. L. (1995) *J. Biol. Chem.* **270**, 1230-1237.

List of Personnel

Christopher J. Green (P. I.)

Xiaoying Chen (original P. I.)

Nahid Waleh

Christopher J. Green

Senior Molecular Biologist
Cell and Molecular Biology Laboratory
Life Sciences Division
SRI International

Specialized Professional Competence

- cDNA subtraction techniques
- Differential display of mRNA differences
- Catalytic RNA design and kinetics
- Genome organization of Gram positive bacteria
- Cloning and expression of human cDNAs coding for enzymes involved in transcription, radiation resistance and nucleotide metabolism
- Cloning of human peptides with anti-arthritis and immune stimulation activities
- Expression of cloned sequences in Baculovirus-insect cell culture, *E. coli* and *B. subtilis*
- Natural enzyme and expressed protein purification
- Expression vector design and construction
- Polymerase chain reaction (PCR) amplification and PCR-mediated site directed mutagenesis
- Extensive DNA and RNA sequencing
- DNA and RNA synthesis and probe design
- Combinatorial chemistry (SELEX)

Academic Background

B.S. (1974) in Biology and Chemistry, University of the Pacific and Ph.D. (1982) in Biochemistry, University of California, Berkeley

PATENTS

Green, C. J. and Johnson, P. H. Cloning and Expression of a Naturally Occurring Variant of Platelet Factor 4 and Compositions Thereof to Modulate Immune Responses. U. S. Patent Number 5,317,011. Filed at the U. S. Patent Office Nov. 9, 1989. Granted May 16, 1994.

Green, C. J. and Johnson, P. H. Cloning and Expression of a Naturally Occurring Variant of Platelet Factor 4 and Compositions Thereof to Modulate Immune Responses. U. S. Patent Number 5,187,075. Filed at the U. S. Patent Office Jan. 26, 1989. Granted Feb. 16, 1993.

Zarling, D., Sena, Elissa, and Green, C. J. Process for Nucleic Acid Hybridization and Amplification. U.S. Patent Number 5,223,414. Filed at the U. S. Patent Office, May 7, 1990. Granted June 29, 1993.

Webster, K., Murphy, B. M., Laderoute, K. L., and Green, C. J. Tissue Specific Hypoxia Regulated Therapeutic Constructs. Submitted

PUBLICATIONS (not including abstracts)

- Green, C. J. The purification and properties of a mammalian tRNA pseudouridine synthetase. Ph.D thesis, University of California, Berkeley, 1982.
- Green, C. J., Kammen, H. O., and Penhoet, E. E. The purification and characterization of a mammalian tRNA pseudouridine synthase. *J. Biol. Chem.* **257**, 3045-3052, 1982.
- Green, C. J., and Vold, B. S. Sequence analysis of a cluster of twenty-one tRNA genes in *Bacillus subtilis*. *Nucleic Acids Research* **11**, 5763-5774, 1983.
- Vold, B. S., and Green, C. J. Transfer RNA gene organization in *Bacillus subtilis*. In *Genetics and Biotechnology of the Bacilli*, A. T. Ganeson and J. A. Hoch (eds.), pp. 35-50. Academic Press, New York. 1984.
- Green, C. J., Stewart, G. C., Hollis, M. A., Vold, B. S., and Bott, K. F. DNA sequence of the *Bacillus subtilis* ribosomal RNA operon, *rrnB*. *Gene*, **37**, 261-266, 1985.
- Vold, B. S. and Green, C. J. Transcription, processing and expression of tRNA genes from *Bacillus subtilis*. In J. A. Hoch and P. Setlow (eds.). *The Molecular Biology of Microbial Differentiation*. American Society for Microbiology, Washington, D.C., pp. 135-141, 1985.
- Vold, B. S. and Green, C. J. Expression of *Bacillus subtilis* tRNA genes in *Escherichia coli* from a promoter within the tRNA gene region. *J. Bacteriol.* **166**, 306-312, 1986.
- Hay, R. E., Tatti, K. M., Vold, B. S., Green, C. J., and Moran, C. P., Jr. Transcription by sigma-29 RNA polymerase of cloned DNA from the *rrnB* region of the *Bacillus subtilis* chromosome. *Gene* **48**, 301-306, 1987.
- Vold, B. S. and Green, C. J. The processing of synthetic tRNA^{His} precursors by the catalytic RNA component of RNase P. *J. Cellular Biochem.* **11C**, 51, 1987.
- Rottmann, W. H., DeSelms, K. R., Niclas, J., Camerato, T., Holman, P. S., Green, C. J., and Tolan, D. R. The complete amino acid sequence of the human aldolase C isozyme derived from genomic clones. *Biochimie* **69**, 137-145, 1987.
- Green, C. J. and Vold, B. S. The processing of tRNA precursors by the catalytic RNA component of RNase P. In *Genetics and Biotechnology of Bacilli*, Vol. II, A. T. Ganesan and J. A. Hoch (eds.), pp. 311-315. Academic Press, Orlando, Florida. 1988.
- Green, C. J. and Vold, B. S. Structural requirements for processing of synthetic tRNA^{His} precursors by the catalytic RNA component of RNase P. *J. Biol. Chem.* **263**, 652-657, 1988.
- Vold, B. S. and Green, C. J. Processing of a multimeric tRNA precursor from *Bacillus subtilis* by the RNA component of RNase P. *J. Biol. Chem.* **263**, 14390-14396, 1988.

Vold, B. S., Okamoto, K., Murphy, B. J., and Green, C. J. Transcriptional analysis of *Bacillus subtilis* rRNA-tRNA operons. I. 21 tRNA genes of *rrnB* have an internal promoter. *J. Biol. Chem.* **263**, 14480-14484, 1988.

Vold, B. S., Green, C. J., Narasimhan, N., Strem, M., and Hansen, J. N. Transcriptional analysis of *Bacillus subtilis* rRNA-tRNA operons. II. Unique properties of an operon containing a minor 5S rRNA gene. *J. Biol. Chem.* **263**, 14485-14490, 1988.

Green, C. J., Vold, B. S., Morch, M. D., Joshi, R. L., and Haenni, A-L. Ionic conditions for the cleavage of the tRNA-like structure of turnip yellow mosaic virus by the catalytic RNA of RNase P. *J. Biol. Chem.* **263**, 11617-11620, 1988.

Green, C. J. and Vold, B. S. Cleavage of the tRNA-like RNA of the turnip yellow mosaic virus by the catalytic RNA component of RNase P. In *Molecular Biology of RNA*, T. Cech, (ed.), pp. 89- 98. Alan R. Liss, Inc., New York, New York, 1989.

Green, C. J., St. Charles, R., Edwards, B.F.P., and Johnson, P. H. Identification and characterization of PF4var1, a human gene variant of platelet factor 4. *Mol. Cell. Biol.* **9**, 1445-1451, 1989.

Waugh, D. S., Green, C. J., and Pace, N. R. The design and catalytic properties of a simplified Ribonuclease P RNA. *Science*, **244**, 1569-1571, 1989.

Green, C. J., Soheli, I., and Vold, B. S. The discovery of new intron-containing human tRNA genes using the polymerase chain reaction. *J. Biol.Chem.*, **265**, 12139-12142, 1990.

Green, C. J. and B. S. Vold. A cluster of nine tRNA genes between ribosomal gene operons in *Bacillus subtilis*. *J. Bacteriol.* **174**, 3147-3151, 1992.

Green, C. J. and Vold, B. S. Transfer RNA, tRNA processing, and tRNA synthetases, p. 683-698. In J. A. Hoch, R. Losick, and A. L. Sonenshein (eds.), *Bacillus and Other Gram Positive Bacteria*. American Society for Microbiology Press, Washington D. C. 1993.

Green, C. J. and Vold, B. S. *Staphylococcus aureus* has clustered tRNA genes. *J. Bacteriol.* **175**, 5091-5096, 1993.

Ausserer, W. A., Bourrat-Floek, B., Green, C. J., Laderoute, K. R., and Sutherland, R. M. Regulation of *c-jun* expression during hypoxic and low-glucose stress. *Mol. Cell. Biol.* **14**, 5032-5042, 1994.

Green, C. J. New Antibiotics Are Needed Against *Staphylococcus*. *Society for Industrial Microbiology News.* **44**, 231-238, 1994.

Rivera-Leon, R., Green, C. J., and Vold, B. S. High-level expression of soluble recombinant RNase P protein from *Escherichia coli*. *J. Bacteriol.* **177**, 2564-2566, 1995.

Green, C. J., Rivera-Leon, R., and Vold, B. S. The catalytic core of RNase P. *Nucleic Acids Research*, **24**, 1497-1503, 1996.

Laderoute, K. R., Knapp, A. M., Green, C. J., Sutherland, R. M, and Kapp, L. N. Expression of the Ataxia Telangiectasia Group D complementing gene in A431 human squamous carcinoma cells. *International Journal of Cancer*, **66**, 772-778, 1996.

Green, C. J. Transfer RNA Gene Organization and RNase P. *Molecular Biology Reports*. **22**, 181- 185, 1996.

Warnecke, J. M., Green, C. J., and Hartmann, R. K. Role of metal ions in the cleavage mechanism by the *E. coli* RNase P holoenzyme. *Nucleosides and Nucleotides* **16**, 721-725, 1997.

B. J. Murphy, G. K. Andrews, A. J. Giaccia, D. J. Discher, C. J. Green, D. Bitte, R. M. Sutherland, J. McCue, K. R. Laderoute, and K. A. Webster. Activation of metallothionein gene expression by hypoxia is regulated through metal response elements and a metal transcription factor-1. *Cancer Res.*, **59**, 1315-1322, 1999.